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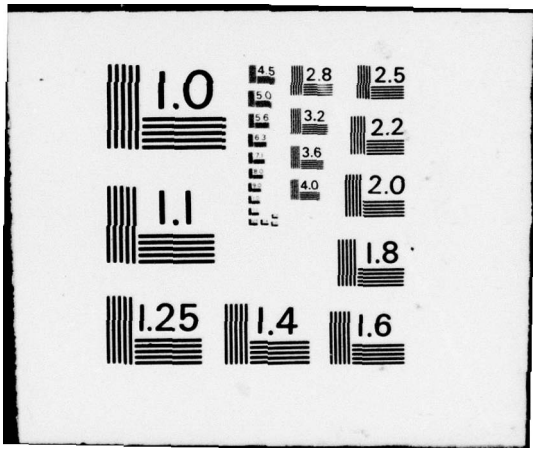
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Crude mediators derived, in vitro, from stimulated rabbit polymorphonuclear leukocytes (LEM) have been shown to engender numerous physiologic and metabolic alterations when injected into normal healthy rats. The LEM-induced changes are similar to those observed during infection in man and laboratory animals. In addition to previous reports of metabolic alterations, these data show that one hour after the intraperitoneal injection of LEM, plasma insulin and glucagon concentrations are elevated; at 2 hours the preceding hormonal alterations are manifested by hepatic metabolic alterations. There is a 30 percent increase in cyclic adenosine monophosphate

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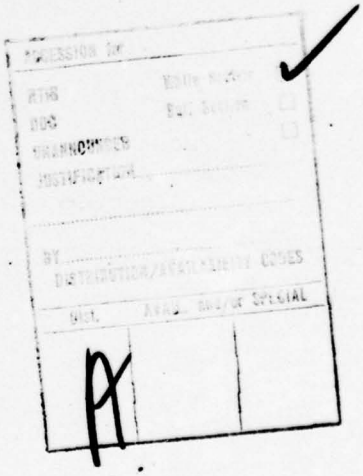
(AMP), glycogen concentrations are depressed, and the concentration of ^{14}C-labeled non-metabolizable amino acid analogs is elevated. The plasma hormone concentrations continue to increase and reach maximum levels by 5 hours, declining to basal by 24 hours. The hepatic concentrations of ^{14}C-labeled nonmetabolizable amino acid analogs parallel the insulin and glucagon responses, increasing through 5 hours then declining to basal by 24 hours. Furthermore, the hepatic concentration of ^{14}C-labeled amino acid analogs is significantly correlated with the inverse of I/G molar ratio. Despite mobilizable of hepatic glycogen, as evident by the depletion of glycogen at 5 hours, plasma glucose concentrations were transiently depressed at 5 hours and returned to basal by 24. Furthermore, dose response studies have been shown that the plasma insulin, glucagon, and hepatic ^{14}C-labeled amino acid analog concentrations are dose dependent 5 hours after an intraperitoneal injection of LEM.

The plasma insulin and glucagon respones to LEM can explain the observed increases in hepatic cyclic-AMP, uptake of ^{14}C-labeled amino acid analogs, and glycogenolysis as well as the relative hypoglycemia. These data partially characterize the role of crude leukcytic mediators in rats, and provide an explanation for the stimuli inducing both hyperglucagonemia and hyperinsulinemia during infection as well as implicating the endocrine pancreas as a factor controlling the host!s metabolic response to infection.



Effect of leukocytic endogenous mediators (LEM) on the secretory response of the endocrine pancreas

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Running title: LEM AND THE ENDOCRINE PANCREAS

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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Abstract:

Crude mediators derived, in vitro, from stimulated rabbit polymorphonuclear leukocytes (LEM) have been shown to engender numerous physiologic and metabolic alterations when injected into normal healthy rats. The LEM-induced changes are similar to those observed during infection in man and laboratory animals. In addition to previous reports of metabolic alterations, these data show that one hour after the intraperitoneal injection of LEM, plasma insulin and glucagon concentrations are elevated; at 2 hours the preceding hormonal alterations are manifested by hepatic metabolic alterations. There is a 30 percent increase in cyclic adenosine monophosphate (AMP), glycogen concentrations are depressed, and the concentration of ^{14}C-labeled nonmetabolizable amino acid anologs is elevated. The plasma hormone concentrations continue to increase and reach maximum levels by 5 hours, declining to basal by 24 hours. The hepatic concentrations of ^{14}C-labeled nonmetabolizable amino acid analogs parallel the insulin and glucagon responses, increasing through 5 hours then declining to basal by 24 hours. Furthermore, the hepatic concentration of ^{14}C-labeled amino acid analogs is significantly correlated with the inverse of I/G molar ratio. Despite mobilization of hepatic glycogen, as evident by the depletion of glycogen at 5 hours, plasma glucose concentrations were transiently depressed at 5 hours and returned to basal by 24. Furthermore, dose response studies have shown that the plasma insulin, glucagon, and hepatic ^{14}C-labeled amino acid analog concentrations are dose dependent 5 hours after an intraperitoneal injection of LEM.

The plasma insulin and glucagon responses to LEM can explain the observed increases in hepatic cyclic-AMP, uptake of ^{14}C-labeled amino acid analogs, and glycogenolysis as well as the relative hypoglycemia. These data partially characterize the role of crude leukocytic mediators in rats, and provide an explanation for the stimuli inducing both hyperglucagonemia and hyperinsulinemia during infection as well as implicating the endocrine pancreas as a factor controlling the host's metabolic response to infection.

Index words: Pancreas; insulin; glucagon; leukocytes; cyclic-AMP

CRUDE SUBSTANCES produced by stimulated rabbit peritoneal polymorphonuclear leukocytes (PMN) induce responses in laboratory animals that are characteriatic of the host's responses to infection. Some of the active components have been partially purified and identified, such as endogenous pyrogen (EP). However, these preparations also contain many additional biologic activities (19) which have been collectively attributed to "leukocytic endogenous mediators" (LEM). When given to healthy rats, LEM depresses plasma zinc and iron concentrations (16,22), increases plasma copper values (16), alters amino acid transport (23,38) and increases synthesis of acutephase globulins (16,23,38). These and other biologic activities of LEM have been summarized (16) and differentiated from those attributed to endotoxin (19,23) or EP (17,19,23).

Comparable alterations are commonly observed during many infections (5,25) and are discussed in a recent review (6). Although many parameters have been examined during infection, little is known about the factors responsible for initiating the catabolic and anabolic responses of the septic host. Numerous investigators have described hyperglucagonemia (10,13,26,27,39), hyperinsulinemia (10,27,28,39), insulin resistance (3,13,26), increased carbohydrate utilization (10,18), altered amino acid metabolism and enhanced protein synthesis (13,24,25). Many of these alterations have been attributed to a substance detected in the plasma of infected man (37) and rats (25), which has biological effects similar to crude LEM.

The observed hyperinsulinemia and/or insulin resistance appears paradoxical in a classical sense. Because severe anorexia generally accompanies sepsis, hypoinsulinemia might be expected similar to that

observed during starvation (2,34). We propose that endocrine alterations are also initiated, by leukocyte factors released into the serum as a direct consequence of the infectious process.

Our data offer a new hypothesis which suggest that the in vivo enhancement of hepatic uptake of amino acids occuring after LEM treatment in rats may be dependent upon an endocrine pancreatic response. Furthermore, these data help to explain the altered glucoregulatory hormone profiles observed during sepsis, as well as the stereotyped patterns of catabolic wasting observed during generalized severe infection.

MATERIALS AND METHODS

Animals. Male, Fisher-Dunning rats weighing 200-250 g were supplied by Microbiological Associates. They were maintained in temperature controlled quarters with 12-h intermittent periods of light (0600-1800 h) and darkness and had ad libitum food and water. Locally purchased New Zealand white rabbits of either sex were used for production of stimulated polymorphonuclear leukocytes (PMN).

Production of mediators. The LEM production procedure was adapted from earlier pyrogen studies (14,17). Peritoneal exudates cells were elicited by intraperitoneal (i.p.) injection of 300-500 ml of 0.2% glycogen (Schwartz-Mann, Orangeburg, N.Y.) in physiological saline into 2-4 kg New Zealand white rabbits. Sixteen hours later the resulting exudate was aspirated from the peritoneal cavity into 800 units of heparin (Abbott Laboratories, North Chicago, Ill.) and passed through sterile gauze. Peritoneal exudates containing more than 95% viable PMN, were processed by a series of steps including: centrifugation at 3,000 rpm in an IEC Model PR-6 centrifuge for 30 min at 4°C; washing with Krebs-Ringer phosphate (KRP) buffer, pH 7.0, containing PSH [2000 units penicillin (Upjohn, Kalamazoo, Mich.), 0.4 mg streptomycin (Charles Pfizer, N.Y.) and 10 units heparin/ml]; osmotic lysis of contaminating red blood cells with sterile water; and repetition of the KRP wash. The washed PMN were resuspended in saline-PSH at a final concentration of 10^8 cells/ml. Following 2 h incubation at 37°C in a shaking water bath, the cells were removed by centrifugation at 15,000 rpm in a Beckman Model L2-65 ultracentrifuge equipped with a type 30 rotor. The supernatant fluid was decanted and filtered through 0.45-μm Nalge filters (Federal Scientific, Kensington, Md.). The filtrate represents the crude LEM preparation used for all experiments.

Bioassay of LEM activities in normal rats. All rats were fasted for 16 h prior to their use in these studies. To determine hepatic amino acid uptake either one of the two ^{14}C-labeled nonmetabolizable amino acid analogs ([1-^{14}C]$_\alpha$-aminoisobutyric acid (AIB) or [1-^{14}C] cycloleucine, New England Nuclear, Boston, Mass.) were employed at a dose of 1 μCi/100 g body wt. Radiolabeled amino acids were injected subcutaneously (s.c.) 16 h before i.p. injection of 1 ml of LEM preparation at 0800 h. Additional groups of rats serving as controls were similarly given 1 ml of inactivated LEM ($_\Delta$LEM, 100°C for 30) or 1 ml of sterile physiological saline. After the injection of LEM, $_\Delta$LEM, or saline into one of the three groups of rats, 6 to 10 rats were sacrificed from each group at selected times during the subsequent 24 h.

At each stated time interval, rats were anesthetized with halothane (Halocarbon Ltd., Malton, Ontario). Portal or peripheral blood samples were collected, and dispensed into two iced test tubes: 2 ml of whole blood were dispensed directly into a tube containing 2000 U Trasylol (F.B.A. Pharmaceuticals, N.Y.) and 3 mg EDTA for the glucagon radioimmunoassay determinations (1), utilizing antibody 30K; and the remainder was dispensed into a heparinized tube. The plasma was separated within 20-30 min after collection by centrifugation at 2000 g for 10 min at 4°C. The heparinized samples were subsequently assayed for immunoreactive insulin (IRI) (15), glucose (31), and zinc (21). All plasma samples were stored at -20°C until assayed.

Two liver samples were obtained in designated studies from each rat. A 1.0 gm portion which was perfused clear of visible blood was used for determination of [^{14}C] content as described earlier (36). A second 5.0 gm liver sample was obtained for subsequent glycogen determinations (30).

Interference of LEM with the radioimmunoassays for insulin and glucagon. Three concentrations of different LEM preparations were substituted directly for plasma samples in both the insulin and glucagon assays. The results of these studies indicate that LEM in concentrations equivalent to or greater than in vivo levels do not interfere with either of the immunoreactive hormone assays.

Dose-response studies were done at 5 h after injection, employing seven doses of LEM which ranged from 0.01-1 ml/100 g body wt and three doses of ΔLEM covering the same range.

Cyclic AMP determinations were performed in 16-h fasted rats, employing LEM, ΔLEM or saline injections. Two hours after injection, 10 rats from each of the three groups were sacrificed and levels of hepatic glycogen and cyclic AMP were determined on liver samples immediately frozen between liquid nitrogen-cooled tongs as described previously (39). Plasma samples obtained at the same time were assayed for immunoreactive glucagon concentrations.

Statistical analyses. Dose-response data were analyzed by regression analysis and the slopes of the control groups (ΔLEM) were compared with the experimental group (LEM) for insulin, glucagon and hepatic amino acid concentrations.

The reciprocal of the insulin/glucagon (I/G) molar ratios were also determined and analyzed as a function of the changing hepatic amino acid concentrations by linear regression analysis using all data points (29).

Statistical analysis on all other data were done using Student's t test for unpaired variates (29).

RESULTS

Fig. 1 illustrates the alterations in plasma glucagon, insulin, glucose and hepatic ^{14}C-labeled amino acid content at various times after an injection of 1 ml of LEM. Plasma glucagon concentrations were significantly elevated at 1 h ($\underline{P}$ <0.001) and continued to increase reaching a maximum level 5 times that of controls at 5 h ($\underline{P}$ <0.001). Plasma concentrations of glucagon declined by 8 h but were still elevated ($\underline{P}$ <0.05) as compared with their 8-h controls, then returned to control values by 24 h (Fig. 1A). Plasma insulin concentrations were also increased in experimental rats and were elevated by 1 h ($\underline{P}$ <0.05). Plasma insulin continued to increase reaching a maximum elevation of 2.8 times control values at 5 h ($\underline{P}$ <0.001), they remained elevated through 8 h ($\underline{P}$ <0.02) declining to basal levels by 24 h (Fig. 1B). Hepatic amino acid flux lagged behind the increases of plasma insulin and glucagon and remained at control levels for the first hour (Fig. 1C). By 2 h hepatic content of ^{14}C-labeled nonmetabolizable amino acid analogs increased ($\underline{P}$ <0.001) to 1.5 times control levels; thereafter, the hepatic amino acid uptake continued to increase peaking at 5 h in a manner which seemed to parallel the hormone elevations. The [^{14}C]amino acid content returned to control levels by 8 h and was lower than controls by 24 h ($\underline{P}$ <0.05), (Fig. 1C). The plasma glucose response was evident only at 5 h ($\underline{P}$ <0.001) at which time glucose was depressed 30% below control values. Plasma glucose concentrations returned to control by 8 h and were depressed ($\underline{P}$ < 0.02) at 24 h (Fig. 1D).

After establishing the time course following a 1.0 ml LEM inoculation, confirmational studies were performed 5 h after LEM, ΔLEM and saline injections. In these studies either portal (Study #1) or

peripheral (Study #2) venous blood samples were obtained, from 2 different groups of rats. These data are presented in Table 1. There were no consistent differences between saline and ΔLEM rats for any of the parameters measured. Therefore, all data were compared to ΔLEM controls. In the first study, portal concentrations of insulin and glucagon were increased in a manner which reduced the average I/G molar ratio. Plasma glucose and zinc were depressed and hepatic concentrations of [1-^{14}C]cycloleucine were increased in the same rats. In Study #2, peripheral plasma concentrations of insulin and glucagon were increased and accompanied by a depression of the average I/G molar ratio. Plasma glucose and zinc were depressed and the hepatic glycogen content was depleted.

After finding that LEM increased plasma glucagon and insulin, increased hepatic uptake of [^{14}C] amino acid and depleted hepatic glycogen stores, studies were performed to determine if there was any correlation between the AIB uptake and cyclic AMP concentrations by 2 h since that was the earliest time that AIB uptake was noted. The data are presented in Table 2. Again, since there was no statistical differences between saline and ΔLEM control values LEM responses were compared to ΔLEM. LEM increased hepatic cyclic AMP 30% ($P < 0.05$), depressed hepatic glycogen and increased peripheral plasma glucagon.

Dose-response studies revealed that the insulin, glucagon, and hepatic amino acid flux were dose-dependent, as shown in Fig. 2A, B, C. Furthermore, there was a postive correlation between the [^{14}C] cycloleucine concentrations and the reciprocal of the I/G molar ratio, $P < 0.001$, as demonstrated in Fig. 2D.

DISCUSSION

Many studies describe the changes occurring in metabolism (10, 20,24,25,35,39) and hormone profiles (10,13,26,27,39) during infection in man and laboratory animals. Although many factors have been implicated as the cause of these alterations, the present demonstration that the i.p. injection of LEM stimulates alterations in most if not all of these parameters including insulin and glucagon, led us to postulate that LEM is capable of initiating hormonal changes which may result in enhanced amino acid and carbohydrate metabolism during infection.

The role of glucagon during infection and following LEM treatment superficiallly appears to be clear, since glucagon can increase cyclic AMP which alters hepatic glycogenolysis, amino acid uptake and gluconeogenesis (8,11,12,32). Data obtained in this study indicated that the hormonal responses result in a significant hepatic cyclic AMP elevation which is similar to earlier reports (10,39). Hormonal and cyclic AMP changes are further manifested by depletion of hepatic glycogen and enhanced amino acid uptake in a dose dependent fashion which correlates with the I/G ratio.

The glucagon response is consistent with the data of Long et al. (18) which show a doubling of the rate of glucose oxidation during sepsis in man. Increased glucose oxidation would require mobilization of available glucose, as well as synthesis from non-carbohydrate sources. Since, amino acids are required by the liver for gluconeogenesis (6,11,33) as well as the synthesis of acute-phase proteins (6,38), the chronic utilization of these essential nutrients would predictably result in negative nitrogen balance and muscle wasting not different from that associated with septic stress (6).

Unlike the glucagon responses the insulin response to infection and LEM in the rat seems paradoxical since one would expect hyperinsulinemia to antagonize the glucagon-mediated effects (12). However, elevation of plasma insulin as observed in this study and others (10,39) does not prevent increased hepatic cyclic-AMP, glycogenolysis or amino acid uptake. In fact, insulin appears to be capable of contributing to some of the responses as implied by the correlation of hepatic amino acid uptake with the I/G ratio and the data of others demonstrating stimulation of hepatic amino acid uptake in vitro by insulin (7).

The mechanism of insulin's action remains controversial and complex as exemplified by reports implicating diverse actions. Included in reports are data indicating insulin can block lipolysis (28), suppress ketone body formation (20), enhance membrane transport of glucose and other nutrients at a rate sufficient to maintain increased glucose oxidation during infection (18). Further evidence of insulin's role was presented by Cherrington and Vranic (9) showing that simultaneous increases of both insulin and glucagon are required to achieve maximum glucose turnover in pancreatectomized dogs.

The plasma glucose responses reported herein support this postulate, since they reflect the rat's ability to mobilize liver glycogen stores and maintain normoglycemia. The reported hypoglycemia following LEM occurs after liver glycogen has been depleted and at the transitional stage from a glucagon predominant to an insulin predominant state. The direct in vivo measurement of hepatic cyclic AMP, glycogenolysis, amino acid uptake, and the hormonal regulators insulin and glucagon in this study, suggest that the LEM induced alterations of plasma glucose, hepatic glycogen and amino acid transport may be secondary to glucagon and

insulin alterations. Although these observations implicate the endocrine pancreas in LEM mediated responses; other reports indicate that they may be due to a direct action of LEM upon the liver, enhancing amino acid uptake in a manner that is neither hormonal nor cyclic AMP dependent (38). An explanation of the differences between the cyclic AMP results when comparing these data with that of others (38) resides in the different experimental designs. The early report (38) conclusively demonstrated that the intraportal injection of LEM did not have a direct affect upon hepatic cyclic AMP. Whereas, our data demonstrate enhanced hepatic cyclic AMP levels by 2 h, presumably due to the hyperglucagonemia at that time.

Although our data indicate that LEM may be acting via the endocrine pancreas, recent reports indicate that 20 µl of LEM injected intracerebroventicularly induces a significant enhancement of hepatic amino acid uptake (4), which may imply a central nervous system mediated response since other routes of administration were ineffective.

In conclusion our data demonstrate that stimulated rabbit peritoneal PMN produce factors which are capable of mimicing certain of the metabolic and hormonal alterations observed during infections. It has been proposed that some of the reported actions of LEM may be mediated by hormonal alterations of glucagon and insulin. Current investigations are directed toward definitively defining the role of these hormones in host metabolic responses to infection.

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TABLE 1. Plasma insulin, glucagon, glucose and zinc and hepatic ^{14}C amino acid analog uptake and glycogen response of rats 5 h after a single i.p. injection of 1 ml of crude LEM

Treatments	Plasma IRI μU/ml	Plasma IRG pg/ml	I/G	Plasma glucose mg/dl	Plasma Zn μg/dl	Hepatic [^{14}C] dpm/mg liver	Hepatic glycogen mg glucose/ liver
Study 1							
Saline (n=8)	17 ± 2	135 ± 20	2.93	121 ± 3	119 ± 6	55 ± 5	-
ΔLEM (n=8)	21 ± 3	275 ± 54	1.76	114 ± 5	94 ± 4	67 ± 3	-
LEM (n=8)	52 ± 4†	1577 ± 186*	0.78	83 ± 5†	51 ± 2*	165 ± 8*	-
Study 2							
Saline	16 ± 4 (n=8)	165 ± 34 (n=9)	2.26	156 ± 4 (n=10)	105 ± 2 (n=9)	-	2.54 ± 0.5 (n=8)
ΔLEM	12 ± 4 (n=6)	104 ± 19 (n=8)	2.69	159 ± 4 (n=10)	96 ± 5 (n=8)	-	2.11 ± 0.1 (n=8)
LEM	36 ± 7† (n=8)	493 ± 28† (n=9)	1.70	125 ± 3† (n=10)	47 ± 1* (n=9)	-	0.28 ± 0.0 (n=8)

Values are means ± SE.

Study 1: Portal plasma vein concentrations of insulin (IRI), glucose, average I/G molar ratios, zinc (Zn) and hepatic uptake of [^{14}C]cycloleucine ([^{14}C]).

Study 2: Peripheral plasma concentrations of insulin (IRI), glucose, average I/G molar ratios, zinc (Zn) and hepatic glycogen.

I/G molar ratio = [(μU insulin/ml)/(pg glucagon/ml)] x 23.33.

*$P < 0.005$, LEM vs. ΔLEM.

†$P < 0.05$, LEM vs. ΔLEM.

TABLE 2. Hepatic cyclic AMP and glycogen and plasma glucagon responses of rats 2 h after a single i.p. injection of 1 ml of crude LEM

Treatment	Hepatic cyclic AMP pmol/mg liver	Hepatic glycogen mg glucose/g liver	Plasma glucagon pg/ml
Saline	0.34 ± 0.02	5.56 ± 1.21	80 ± 11
ΔLEM	0.36 ± 0.02	6.22 ± 0.51	96 ± 11
LEM	0.46 ± 0.02	2.81 ± 0.46*	222 ± 40†

Values are mean ± SE, of 10 rats.

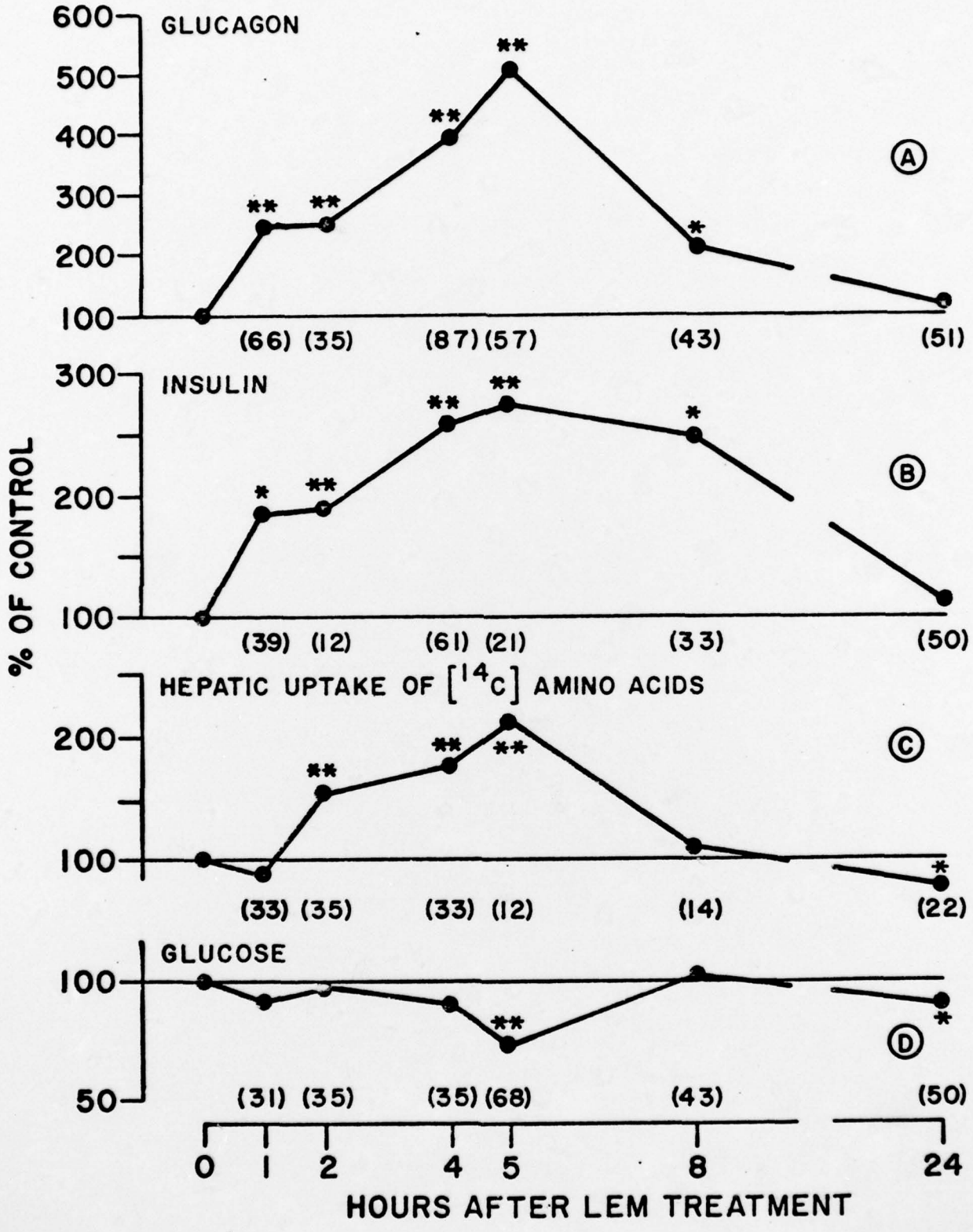
*P < 0.05, LEM vs. ΔLEM.

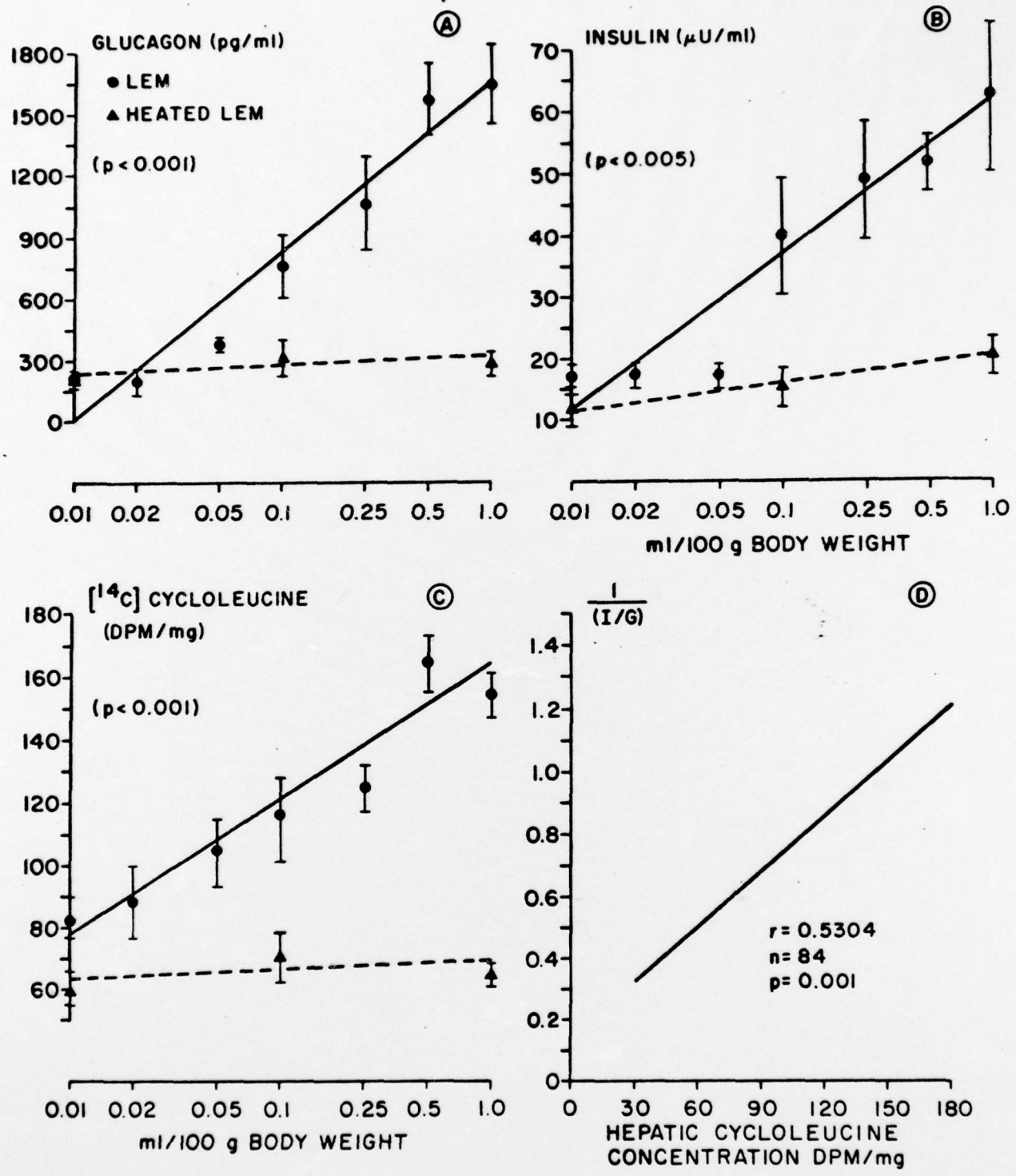
†P < 0.005, LEM vs. ΔLEM.

LEGENDS TO FIGURES

FIG. 1. Data represent a composite of results obtained with several lots of crude LEM, and are expressed as a percent of their respective controls. To standardize these data the control values from saline and heat-inactivated LEM preparations were averaged for each parameter at each time; then the individual values were expressed as a percent of their respective means, t tests for unpaired variates were performed to determine if there were differences between saline controls and heat-inactivated ΔLEM-treated control groups. Since there were no statistical differences, the control data were combined and used as a single control group for each time point. Each LEM-treated data point was then expressed as a percent of its respective control. Thereafter the percents were analyzed as discrete values by Student's t test for unpaired variates. *P <0.05 as compared to the controls at the same time. **P <0.005 as compared to the controls at the same time. Numbers in parenthesis indicate the number of observations at each time point.

FIG. 2. Linear regression analysis of the glucagon (A), insulin (B) and [^{14}C]cycloleucine (C) concentrations are plotted against the log doses of LEM. Each point represents the mean ± SE of 10 animals (•-C). Significance of the differences between the slopes for LEM and ΔLEM-treated rats are shown. The reciprocal of the I/G molar ratio is plotted as a function of the hepatic cycloleucine concentration (D). Correlation coefficient (r), n and P are shown.


GLUCAGON
600
500
400
300
200
100
(66) (35) (87) (57) (43) (51)
A
INSULIN
300
200
100
(39) (12) (61) (21) (33) (50)
B
% OF CONTROL
HEPATIC UPTAKE OF [14C] AMINO ACIDS
200
100
(33) (35) (33) (12) (14) (22)
C
GLUCOSE
100
50
(31) (35) (35) (68) (43) (50)
D
0 1 2 4 5 8 24
HOURS AFTER LEM TREATMENT


A
GLUCAGON (pg/ml)
● LEM
▲ HEATED LEM
(p < 0.001)
1800
1500
1200
900
600
300
0
0.01 0.02 0.05 0.1 0.25 0.5 1.0
B
INSULIN (μU/ml)
(p < 0.005)
70
60
50
40
30
20
10
0.01 0.02 0.05 0.1 0.25 0.5 1.0
ml/100 g BODY WEIGHT
C
[14C] CYCLOLEUCINE
(DPM/mg)
(p < 0.001)
180
160
140
120
100
80
60
0.01 0.02 0.05 0.1 0.25 0.5 1.0
ml/100 g BODY WEIGHT
D
1/(I/G)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
r = 0.5304
n = 84
p = 0.001
0 30 60 90 120 150 180
HEPATIC CYCLOLEUCINE
CONCENTRATION DPM/mg